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(54) Title: ANAPLASTIC LYMPHOMA KINASE ASSAY, REAGENTS AND COMPOSITIONS THEREOF

(57) Abstract: Disclosed are methods for detecting ALK tyrosine kinase activity and for the identification of compounds that modulate ALK activity, peptide substrates, reagents and compositions thereof.

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# ANAPLASTIC LYMPHOMA KINASE ASSAY, REAGENTS AND COMPOSITIONS THEREOF

The present invention provides an assay for measuring the kinase activity of the Anaplastic lymphoma kinase (ALK). More specifically the invention is directed to methods, reagents and compositions for detecting the tyrosine-phosphorylating activity of ALK. The assay is particularly useful for in vitro screening and identification of potential ALK inhibitors.

## BACKGROUND OF THE INVENTION

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase, believed to play an important role in the development and function of the nervous system. ALK is normally expressed in the central nervous system, with peak expression during the neonatal period. However, due to chromosomal translocations, ALK is also aberrantly expressed and activated in some cancers in the form of oncogenic fusion proteins. ALK fusion proteins are responsible for approximately 5 - 10% of all non-Hodgkin's lymphomas. The annual incidence of ALK positive lymphomas is about 100 000 worldwide, with 2000 – 3000 new cases occurring in EU countries. ALK is an excellent candidate for therapeutic intervention, as it plays an essential role in oncogenicity and its normal expression is mostly restricted to the central nervous system. Hence, a specific ALK inhibitor could be an efficient treatment for ALK positive lymphomas with few associated clinical side effects. For the identification of potential ALK inhibitors it is highly desirable to develop a suitable assay for assessing directly the inhibitory effect of compounds on enzyme activity.

## DESCRIPTION OF THE INVENTION

According to the invention, an *in vitro* kinase assay specific for ALK and useful for screening compounds that modulate ALK activity is provided.

This assay is based on the use of a peptide substrate which is readily phosphorylated by ALK and on the subsequent detection of the phosphorylated product thus obtained. In a preferred embodiment of the invention the assay is an ELISA wherein the phosphorylated product is detected by immunochemical reactions.

In order to generate a selective ALK substrate, peptides reproducing the sequence of ALK activation loop (aa 1274-1294: ALK_HUMAN, Q9UM73, swiss-PROT) were synthesized and tested. The peptides ARDIYRASFFRKGGCAMLPVK (SEQ ID N. 1) and ARDIYRASYYRKGGCAMLPVK (SEQ ID N.2) were particularly effective as ALK substrates showing a phosphorylation degree higher than that of polyGlu/Tyr, a random polymer which is known to be a good substrate for most tyrosine kinases. The first object of the invention is therefore a peptide having an amino acid sequence selected from SEQ ID N. 1 and SEQ ID N. 2.

A further object of the invention is a method for detecting ALK tyrosine kinase activity, which essentially comprises the steps of:

i) incubating the ALK protein or a functional derivative thereof with a peptide selected from SEQ ID N. 1 and 2 in conditions suitable for phosphorylation of the peptide;
ii) detecting the phosphorylated peptide thus formed.

As used herein, "ALK functional derivative" means any modified form of ALK protein, for example a truncated or conjugated form or a fragment thereof, which maintains the catalytic activity of unmodified ALK. The functional derivative should preferably contain the entire catalytic domain of ALK spanning residues 1116-1392 of ALK sequence (Q9UM73). The portion of ALK protein stretching from residue $Leu^{1073}$ to $Ala^{1459}$ is preferably used. When produced by recombinant gene technology using the baculovirus-based expression system, this ALK fragment shows a correct folding (confirmed by

CD spectra) and an effective catalytic activity.

In addition, a preparation containing a constitutively active form of ALK may be used instead of the purified protein or its functional derivative. Such a preparation is preferably a cell lysate, which could be used to a) assess for ALK activity in whole cells expressing ALK or ALK fusion proteins and b) assess for the effect of potential inhibitors on ALK within a cell, by treating ALK-expressing cells with different compounds and then assaying cell lysate for ALK kinase activity.

To detect the phosphorylated peptide in step ii), the phosphorylation reaction of step i) can be performed in the presence of a radioactive reagent, such as [$\gamma^{32}$P]ATP or [$\gamma^{33}$P]ATP, whereby a radioactive product is formed which is easily detected by radiometric techniques. Alternatively, an immunoreaction can be carried out, which comprises the formation of an immune complex between the phosphorylated peptide and an anti-phosphotyrosine antibody. This anti-phosphotyrosine antibody can be radioactively labelled or conjugated to a reporter enzyme, such as horse-radish peroxidase, beta-galactosidase, alkaline phosphatase or glucose oxidase, thereby allowing the direct detection of the antibody and thus of the phosphorylated peptide by measuring the specific radioactivity or enzymatic activity. As a further alternative, the anti-phosphotyrosine antibody may be detected indirectly by a second antibody recognizing the anti-phosphotyrosine antibody and carrying a radioactive label or a reporter enzyme, or by an enzyme conjugated streptavidin which recognises a biotin label on the anti-phosphotyrosine antibody.

In a preferred embodiment the invention provides a method for detecting ALK tyrosine kinase activity which comprises the steps of:

a) adhering a peptide of SEQ ID N. 1 or 2 to a solid phase;

b) incubating the solid phase with an ALK fragment extending from

Leu$^{1073}$ to Ala$^{1459}$ in suitable conditions for tyrosine phosphorylation;

c) washing the solid phase;

d) incubating the solid phase with an anti-phosphotyrosine antibody (primary antibody) in conditions suitable for antigen-antibody binding;

e) washing the solid phase;

f) incubating the solid phase with an enzyme-conjugated antibody (secondary antibody) recognizing the anti-phosphotyrosine primary antibody in conditions suitable for binding of primary antibody to secondary antibody, so that a ternary immune complex is formed;

g) washing the solid phase;

h) measuring the enzymatic activity of the immune complex wherein the measured activity is proportional to the amount of tyrosine-phosphorylation.

This assay, which is properly an ELISA-based kinase assay, utilizes enzyme-antibody conjugates. The conjugated enzyme cleaves a substrate to generate a colored reaction product that can be detected spectrophotometrically by measuring the absorbance of the colored solution, which is proportional to the amount of phosphotyrosines.

Solid phases or supports which can be used according to the invention comprise plastic material (reaction plates, wells, vials), polystyrene, latice and magnetic beads, colloidal metal particles, glass and/or silica surfaces and others.

The ELISA-based ALK assay is preferably used for the screening of compounds that modulate the tyrosine-phosphorylating activity of ALK, in particular for screening ALK inhibitors.

In a preferred embodiment the invention is therefore directed to a

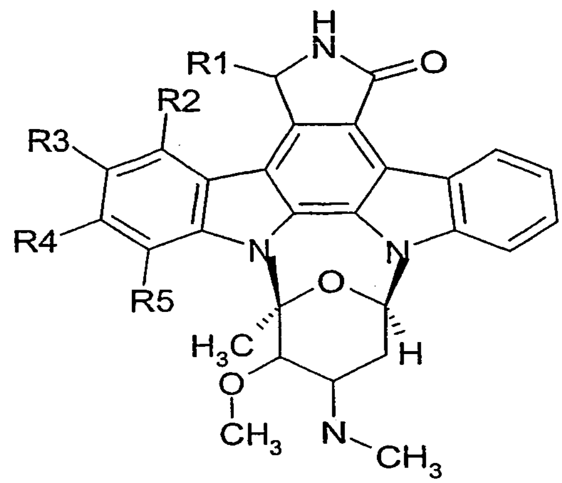
method for the identification of compounds that modulate ALK tyrosine-kinase activity, wherein the ALK assay described above is carried out in the presence of a candidate compound or of a compound known to stimulate or inhibit ALK tyrosine kinase activity (control). Specifically, the method for identifying compounds that modulate ALK tyrosine-kinase activity comprises the steps of

i) incubating ALK protein or a functional derivative thereof with a peptide selected from SEQ ID N. 1 or 2, preferably SEQ ID N. 1, in the presence of a candidate compound, in conditions suitable for phosphorylation of the peptide;

ii) quantifying the phosphorylated peptide thus formed;

Compounds that inhibit ALK activity are selected as potential therapeutic agents for use in the treatment of ALK-related tumors, such as anaplastic large cell lymphomas and non-Hodgkin lymphomas. The ALK-modulating activity of a candidate compound can be compared to that of a reference compound (control), which is assayed under the same conditions as the candidate compound. Staurosporine (Meggio F. et al, Eyr. J. Biochem. (1995) 234, 317-322), which proved effective as an ALK inhibitor at 0.2 – 0.3 μM concentration, can be used as a positive control when screening other compounds for ALK inhibitory activity.

Studies of molecular modeling gave important indications as to the pattern of substitutions required to improve staurosporine affinity and specificity towards ALK protein. The most effective structures are represented in the following general formula (I):



wherein R1 and R2, independently of one another, are selected from halogen, preferably chlorine, phenyl or C1-C3 alkyl optionally substituted with one or more halogens; R3 is hydroxyl; R4 is hydroxyl or hydroxymethyl; R5 is C1-C3 alkyl, optionally halo-substituted, or benzyl.

The compounds of formula (I) antagonize the binding of ATP to the tyrosine kinase domain of ALK within oncogenic fusion proteins such as NPM-ALK, ATIC-ALK, CTLC-ALK, TFG-ALK or other sporadic fusions with tropomyosins. Accordingly, they can be used in the preparation of a medicament for the treatment of ALK-related tumors, especially anaplastic large cell lymphomas and non-Hodgkin lymphomas.

In a different embodiment of the invention, compounds (I) are used in an ALK-assay for the screening of ALK-inhibitors, as herein disclosed. High-throughput screenings can also be implemented using the ALK assay of the invention.

According to a further aspect, the invention is directed to a kit for carrying out the ALK assay described above. The kit consists of a packaged combination of reagents in predetermined ratios. Typically the kit will contain a peptide of SEQ ID N: 1 or 2, optionally immobilized on a solid phase, the anti-phosphotyrosine antibody and, if necessary, an antibody labelled with an enzymatic or radioactive label. In addition the kit may contain reagents for

colorimetric or radiometric reactions, buffers, controls such as staurosporine or a derivative thereof, diluents, detergents, stabilizers and any other component useful for setting up and carrying out the assay. The relative amounts of the various reagents may be varied to optimize the sensitivity of the assay. Particularly the reagents may be provided in solid or liquid preparations, such as solution, suspension, dispersion, dry powders, lyophilized preparation. The kit components are supplied in single or separate containers. The kit may also include instructions for carrying out the assay.

## DESCRIPTION OF THE FIGURES

**Figure 1**: purification and activity of recombinant His-tagged ALK

A) Silver stained 10% SDS-PAGE gel showing marker (M), dialysed and pooled DEAE column fractions loaded onto the HiTrap column (load), HiTrap column fractions (numbers indicate fraction number). B) Radioactive autophosphorylation assay of purified rALK. Lane 1: autoradiograph of 32P-labelled rALK. Lane 2: silver staining of the same sample as in Lane 1.

**Figure 2**: kinetics for ALK kinase with peptides

**Figure 3**: Detection of rALK activity using the ELISA based kinase assay

A) Phosphorylation of peptide SEQ ID N.2 by purified rALK. An ELISA kinase reaction was performed with or without 0.5 μg purified rALK, 15 μg peptide SEQ. ID N.2 or no peptide, at 30°C for 30 mins. The graph shows absorbance at 450 nm. B) The kinetics of ALK peptide substrate phosphorylation. An ELISA kinase reaction was performed using 206 μM peptide SEQ. ID N.2 or 42 μM peptide SEQ. ID N.1 with 0.1 μg of purified rALK. The reaction was stopped by adding EDTA after 0, 0.5, 2, 5, 10 and 15 mins.

**Figure 4**: The effect of peptide substrate concentration on level of phosphorylation

The ELISA assay was performed in the presence of 0.2 µg purified rALK and 0 - 105 µM peptide SEQ ID N.1 (A) or 0 - 315 µM peptide SEQ ID N.2 (B), at 30°C for 15 mins.

**Figure 5**: The effect of rALK concentration on substrate phosphorylation

An ELISA assay was performed using 206 µM peptide SEQ ID N.2 or 42 µM peptide SEQ ID N.1 and rALK ranging from 0 – 225 ng, at 30°C for 15 mins.

**Figure 6**: Inhibition of rALK activity by Staurosporine

An ELISA assay was performed using 42 µM SEQ ID N.1, 20 ng rALK, at 30°C for 15 mins in the presence of 0 – 5 µM staurosporine or an equivalent volume of solvent, DMSO. Graph shows A450 normalised to the untreated control.

## EXPERIMENTAL RESULTS

### Peptide synthesis

Peptides were synthesised by automatic solid phase synthesis utilising 9-fluorenilmethoxycarbonyl (Fmoc) chemistry with an Applied Biosystems Model 431A synthesiser on 4-hydroxymethyl-copolystirene-1% divinylbenzene-resin (0.95 mmol/g, 0.05 mmol).

Fmoc amino acids (0.5 mmol) were activated by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1 eq) and 1-hydroxybenzotriazole (HOBt) (1 eq) in the presence of DIPEA (2 eq).

Peptidyl-resins (100 mg) were cleaved and deprotected in a mixture containing 5 ml of trifluoroacetic acid, 0.375 gr of phenol, 0.125 µl of 1,2-ethanedithiol, 0.250 µl of thioanisole, and 0.25 µl of water for 2 hours. The reaction mixture was filtered onto a tube containing ethyl ether cooled at 0°C. Precipitated peptides were separated by centrifugation and washed with fresh ether.

Crude peptides (50-100 mg in 10 ml of water) were pumped onto a preparative RP-column (prepNova-Pak HR C18, 6 µm, 25x10 mm, Waters) and eluted with a linear gradient of 10% to 45% acetonitrile at 12 ml/min.

The purity of peptides was > 90% as judged by analytical RP-HPLC on a 5 µm, C18 Symmetry300 column, 4.6x250 mm (Waters) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min. Peptide molecular weights were confirmed by mass spectroscopy using a matrix-assisted laser-desorption ionisation time-of-flight (MALDI-Tof) spectrometer (Maldi-1; Kratos-Schimadzu, Mancester, UK).

**Production and purification of recombinant ALK**

A portion of ALK, stretching from amino acids $Leu^{1073}$ to $Ala^{1459}$, has been cloned into the baculovirus transfer vector, pBlueBacHis2C (Invitrogen). Using this vector and the MaxBac® 2.0 baculovirus expression system (Invitrogen) we have produced recombinant baculovirus and expressed recombinant ALK (rALK) protein in Sf9 (*Spodoptera frugiperda*) insect cells. This protein has a theoretical molecular weight of 49 kDa and contains the predicted catalytic domain of ALK ($Ile^{1116}$ to $Val^{1392}$) fused to a 6-histidine-tag (His-tag). The autophosphorylation activity of His-tagged rALK, which is indicative of correct folding, has been verified by anti-phosphotyrosine immunoblotting of whole cell lysates and an *in vitro* radioactive kinase assay.

To produce protein for purification, Sf9 cells were infected with recombinant virus at a multiplicity of infection (MOI) of 5. Cultures were incubated for 3 days at 27°C and then harvested by centrifuging at 400 g for 10 minutes at 4°C. Cell pellets were stored at -80°C until use. To lyse the cells, cell pellets were resuspended in hypotonic buffer (Buffer A) containing 50 mM Tris-HCl pH 8, 20 mM NaCl and protease inhibitors (Pepstatin, Benzamidine, Leupeptin and Aprotinin). After 30 minutes incubation on ice, the cell suspension was centrifuged at 1500 g for 15 minutes at 4°C and the

supernatant was filtered through a 0.45 μm filter. The filtrate was then loaded onto an 80 ml anion exchange Fast Flow DEAE-sepharose (Sigma) column in the presence of Buffer A at a flow of 1.5 ml/min using the AKTA FPLC system (Amersham-Pharmacia Biotech). Bound proteins were eluted in gradient of NaCl ranging from 20 to 200 mM. Fractions were analysed for the presence of ALK protein by immunoblotting. Positive fractions were pooled and dialysed for 3 h at 4°C in 1 litre of native binding buffer (20 mM sodium phosphate pH 7.8, 500 mM NaCl and PI), changing the buffer every hour. Dialysed fractions were then loaded onto a HiTrap™-nickel affinity chromatography column (Amersham-Pharmacia Biotech), previously equilibrated with native binding buffer supplemented with 50 mM imidazole and 20 mM $\beta$-mercaptoethanol. A flow of 0.5 - 1 ml/min was applied to the column. After washing the column with native binding buffer bound proteins were eluted using a linear gradient of imidazole ranging from 50 to 200 mM. Fractions were then analysed for the presence and purity of rALK by SDS-PAGE and silver staining. Figure 1A shows an example purification of rALK.

Finally, positive fractions were dialysed in 50 mM Tris pH 7.4, 100 mM NaCl, 10% glycerol, 20 mM β-mercaptoethanol and protease inhibitors, and stored at –80°C until use. To verify that purified rALK was active we have performed a radioactive autophosphorylation assay. A reaction mix containing 25 μl of a rALK positive fraction, 6 μM cold ATP, 1.2 mM DTT, 10 μCi [$\gamma$-$^{32}$P]ATP, 25 mM Hepes pH 7.5, 10 mM $MgCl_2$ and 10 mM $MnCl_2$ was incubated at 30°C for 30 minutes. The reaction was stopped by adding Laemmli buffer and heating at 95°C for 5 minutes. The samples were resolved on a 10% SDS-PAGE gel and transferred to an Immobilon™-P membrane. Radioactively labelled proteins were visualized by autoradiography as shown in Figure 1B.

**Peptide phosphorylation (radioactive assay)**

Peptides and the random polymer polyGlu/Tyr (1:4) were phosphorylated in 30 μl of a medium containing 50 mM Tris/HCl, pH 7.5, 5 mM $MnCl_2$, 10 μM Na-vanadate, 30 μM [$\gamma^{32}$P]ATP or [$\gamma^{33}$P]ATP (specific activity 1000 cpm/pmol) and 10 units of rALK [one unit was defined as the amount of enzyme transferring 1 pmol phosphate per min to polyGluTyr (0.1 mg/ml)]. The reactions were terminated after 10 min of incubation at 30°C by spotting 25 μl of the mixture onto P81 phosphocellulose papers, which were processed as described elsewhere (1). Kinetic constants were determined by GraphPad Prism software fitting the data directly to the Michaelis-Menten equation using nonlinear regression.

***Results***

The peptide derived from ALK reference sequence, containing Tyr-1278 was a good substrate of rALK, whereas peptides containing either Tyr-1282 or Tyr-1283 were slightly affected by the enzyme (see Fig. 2). The Tyr-1278-derivative was phosphorylated with an efficiency even higher than that displayed by the peptide bearing three Tyr residues (Table I). To confirm that Tyr-1278-derivative is an optimal peptide substrate for ALK catalytic domain, we compared its phosphorylation degree with that displayed by polyGlu/Tyr (1:4), a random polymer that is a very good substrate for most tyrosine kinases. Table II shows that Tyr-1278-derivative is a better ALK substrate than polyGlu/Tyr.

TABLE I

Kinetic constants for rALK with synthetic peptides.

| PEPTIDE | $V_{max}$ (pmol/min) | $K_m$ (μM) | Efficiency ($V_{max}/K_m$) |
|---|---|---|---|
| ARDIYRASYYRKGGCAMLPVK | 99.5 | 90.5 | 1.1 |
| ARDIYRASFFRKGGCAMLPVK | 186.3 | 109.4 | 1.7 |

TABLE II

Phosphorylation rates of model substrates by ALK catalytic domain.

Poly(Glu/Tyr) and peptide concentrations were 0.1 mg/ml and 400 μM, respectively. Enzyme concentration was 10 units. Reported values represent the means for three separate experiments. S.E.M. values were always less than 14%.

| SUBSTRATE | Phosphorylation degree (pmol/min) |
|---|---|
| Poly(Glu/Tyr) | 10.0 |
| ARDIYRASFFRKGGCAMLPVK | 30.3 |

**ELISA-based *in vitro* kinase assay**

A Nunc Immuno 96 well plate was incubated overnight at 37°C with coating solution (125 μl/ well) containing ALK peptide substrate (SEQ ID N. 1 or SEQ ID N. 2) at various concentrations in PBS. The wells were then washed with 200 μl of wash buffer (PBS-Tween 0.05 %) and left to dry for at least 2 hours at 37°C. The kinase reaction was performed by incubating kinase buffer (25 mM Hepes pH 7.5, 5 mM $MnCl_2$, 5 mM $MgCl_2$), 0.3 mM ATP and purified rALK at various concentrations in a total volume of 100 μl/well at 30°C for 15 minutes. The reaction mix was then removed and wells were washed 5 times with 200 μl of wash buffer. Phosphorylated peptide was detected using 100 μl/well of a mouse monoclonal anti-phosphotyrosine antibody (clone 4G10 UpstateBiotech Ltd) diluted 1:500 in PBS + 4% BSA. After 30 minutes incubation at room temperature the antibody was removed and wells were washed as described above. 100 μl of a secondary antibody (anti-mouse IgG, Horseradish Peroxidase linked whole antibody, from sheep, Amersham Pharmacia Biotech) diluted 1:1000 in PBS + 4% BSA was added to each well and the plate was incubated again for 30 minutes at room temperature before washing as above. The plate was developed using 100

μl/well TMB Substrate Solution (Endogen) and the reaction was stopped by adding an equal volume of $H_2SO_4$ 0.18 M. Finally, the absorbance was read at 450 or 490 nm using an Ultrospec® 300 spectrophotometer (Amersham-Pharmacia Biotech).

***Results***

We have shown that the ELISA-based kinase assay can be used to detect phosphorylated ALK peptide substrates (SEQ ID N.1 and N.2) and hence the activity of purified rALK. In initial experiments we performed the assay in the presence and absence of both purified rALK and the peptide substrate SEQ ID N.2, in order to determine if a specific increase in absorbance at 450 nm (A450) could be observed (Figure 3A). Indeed in the presence of both rALK and substrate a dramatic increase in A450 was observed. In contrast, in the absence of either peptide or rALK or both, A450 was low, indicating a low level of background absorbance. To determine the kinetics of substrate phosphorylation we measured A450 after various incubation times (Figure 3B). For both peptides the kinetics of the reaction was similar with maximum phosphorylation achieved after 10 minutes.

To compare the efficiency of the two peptides in the ELISA kinase assay and to determine the optimal concentration of peptide to use we performed standard curves for both peptides. Results show that increasing the amount of peptide substrate increased A450 and hence rALK kinase activity. For the peptide substrate SEQ ID N.2, maximum A450 and hence phosphorylation was observed at approximately 200 μM, compared with only 25 μM for peptide substrate SEQ ID N.1 (Figures 4A and B). This indicates that peptide substrate SEQ ID N.1 is more efficient as a substrate for rALK.

To observe inhibition of kinase activity it is essential that we use an appropriate concentration of enzyme, i.e. in the linear range of a standard

curve. An ELISA was performed using 206 μM SEQ ID N.2 or 42 μM SEQ ID N.1 and rALK ranging from 0 – 220 ng. The linear range of the curve for both substrates was approximately between 0 – 15 ng of rALK, after which the curves reached plateau (Figure 5).

The effect of the inhibitor staurosporine on rALK activity was assessed using 42 μM peptide substrate SEQ ID N.2. A solvent control containing DMSO at corresponding concentrations was also performed. Staurosporine markedly inhibited rALK activity reaching maximum inhibition at 1 μM and with an estimated $IC_{50}$ = 300 nM (Figure 6). The solvent (DMSO) alone had no substantial effect on rALK activity.

## REFERENCES

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## CLAIMS

1. A method for detecting ALK tyrosine kinase activity, which comprises the following steps:

   i) incubating the ALK protein or a functional derivative thereof with a peptide substrate selected from SEQ ID N. 1 or 2 in conditions suitable for phosphorylation of the peptide;

   ii) detecting the phosphorylated peptide.

2. A method according to claim 1, wherein the peptide has sequence SEQ ID N. 1.

3. A method according to claim 1, wherein purified ALK protein or an ALK-containing preparation is used.

4. A method according to claim 3, wherein said preparation is a cell lysate.

5. A method according to claim 1, wherein said functional derivative contains the entire catalytic domain of ALK spanning residues 1116-1392 of ALK sequence.

6. A method according to claim 5, wherein said functional derivative is a fragment of ALK protein extending from residue $Leu^{1073}$ to $Ala^{1459}$.

7. A method according to claim 6, which comprises the steps of:

   a) adhering a peptide of SEQ ID N. 1 or 2 to a solid phase;

   b) incubating the solid phase with said ALK fragment in conditions suitable for tyrosine phosphorylation;

   c) washing the solid phase;

   d) incubating the solid phase with an anti-phosphotyrosine antibody (primary antibody) in conditions suitable for antigen-antibody binding;

   e) washing the solid phase;

   f) incubating the solid phase with an enzyme-conjugated antibody

(secondary antibody) recognizing the primary antibody in conditions suitable for the binding of primary and secondary antibodies, so that a ternary immune complex is formed;

g) washing the solid phase;

h) measuring the enzymatic activity of the immune complex wherein the measured activity is proportional to the amount of tyrosine-phosphorylation.

8. A method according to claim 7, wherein the enzyme conjugated to the antibody is Horse-Radish peroxidase.

9. A method according to claim 7, wherein the enzymatic activity is detected by colorimetric reaction.

10. A method according to any previous claims, for the identification of compounds that modulate ALK tyrosine-kinase activity.

11. A method according to claim 10, which comprises the steps of

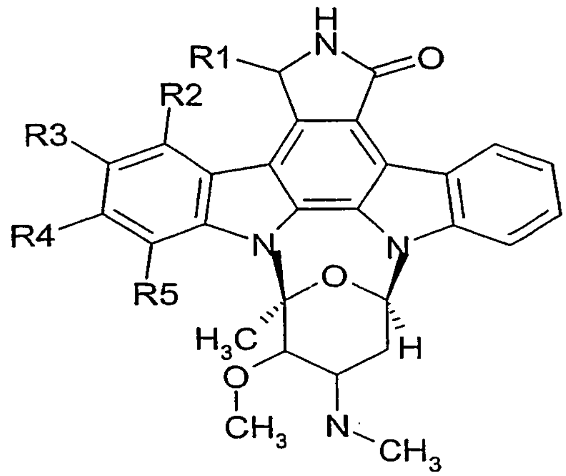
i) incubating ALK protein or a functional derivative thereof with a peptide selected from SEQ ID N. 1 or 2 in the presence of a candidate compound (a) in conditions suitable for peptide phosphorylation;

ii) detecting the phosphorylated peptide thus formed;

12. A method according to claims 10-11, wherein the ALK-modulating activity of the candidate compound is compared to that of a reference compound which is assayed under the same conditions as the candidate compound.

13. A method according to claim 12, wherein the reference compound is staurosporine.

14. A method according to claim 12, wherein the reference compound is a staurosporine derivative of general formula (I):




wherein R1 and R2, independently of one another, are selected from halogen, preferably chlorine, phenyl or C1-C3 alkyl optionally substituted with one or more halogens; R3 is hydroxyl; R4 is hydroxyl or hydroxymethyl; R5 is C1-C3 alkyl, optionally halo-substituted, or benzyl.

15. A peptide useful as ALK substrate selected from SEQ ID N. 1 or 2.

16. A peptide acording to claim 15, which is SEQ ID N. 1.

17. The use of a peptide according to claim 15 or 16 for the determination of ALK tyrosine-kinase activity.

18. The use of a compound of formula (I), as per claim 14, for the preparation of a medicament for the treatment of ALK-related tumors, especially anaplastic large cell lymphomas and non-Hodgkin lymphomas.

19. A kit for detecting ALK tyrosine-kinase activity according to claims 1-14, which comprises a peptide of SEQ ID N: 1 or 2 and an anti-phosphotyrosine antibody.

20. A kit according to claim 19, containing an additional component selected from reagents for colorimetric reactions, buffers, diluents, detergents, stabilizers, staurosporine or a derivative thereof as per claim 14.

Fig 1

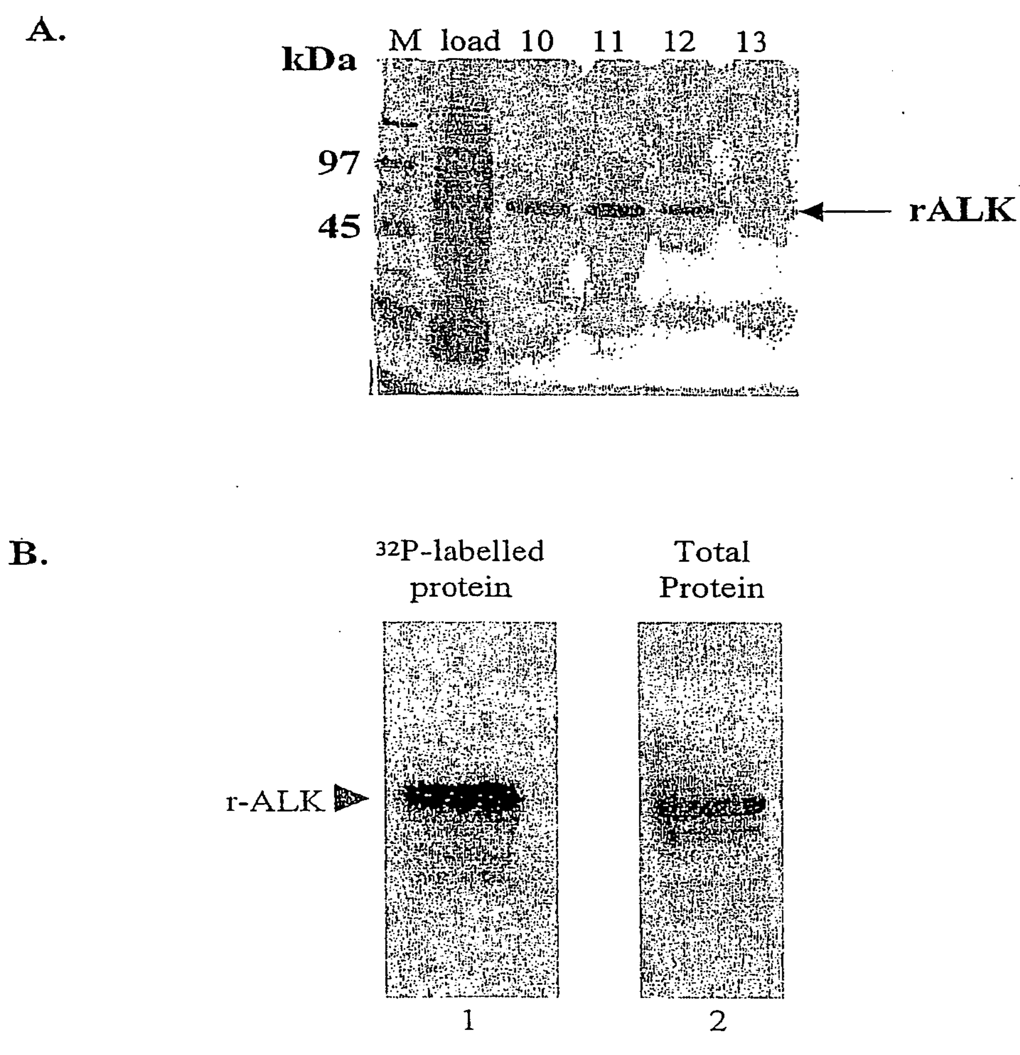

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Fig 2

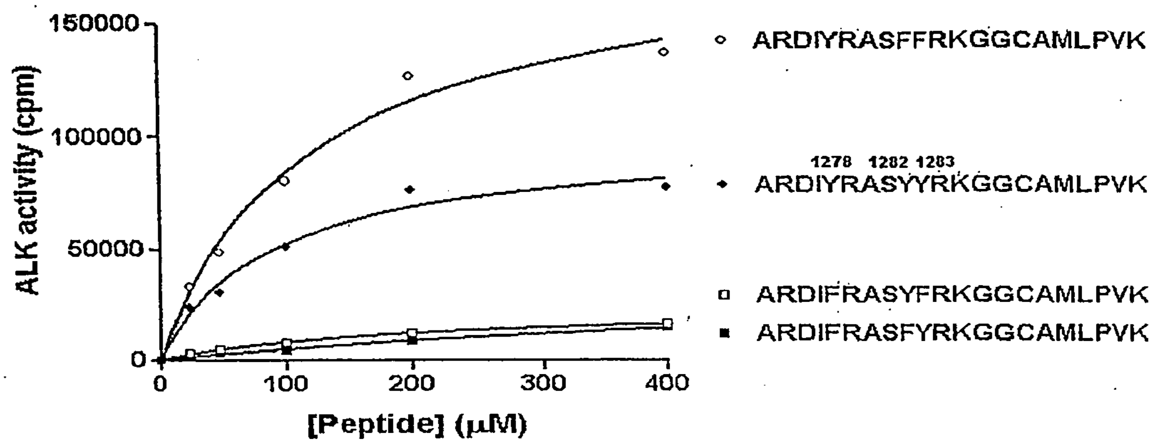
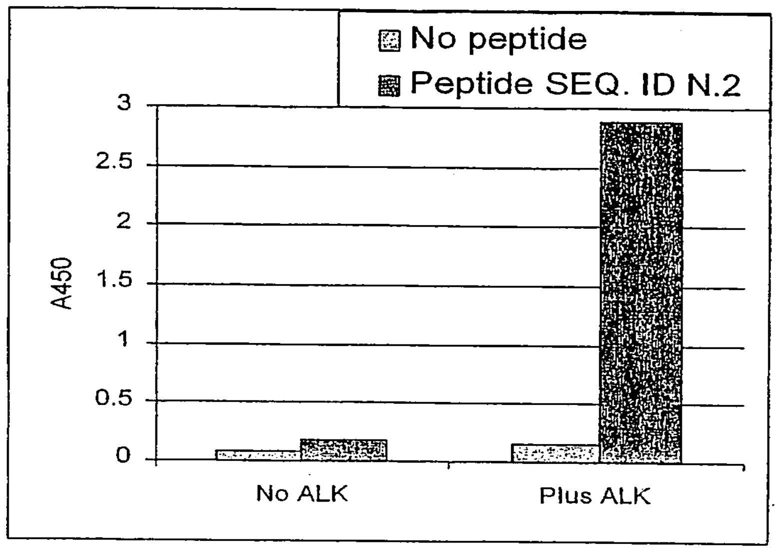


Fig 3

A.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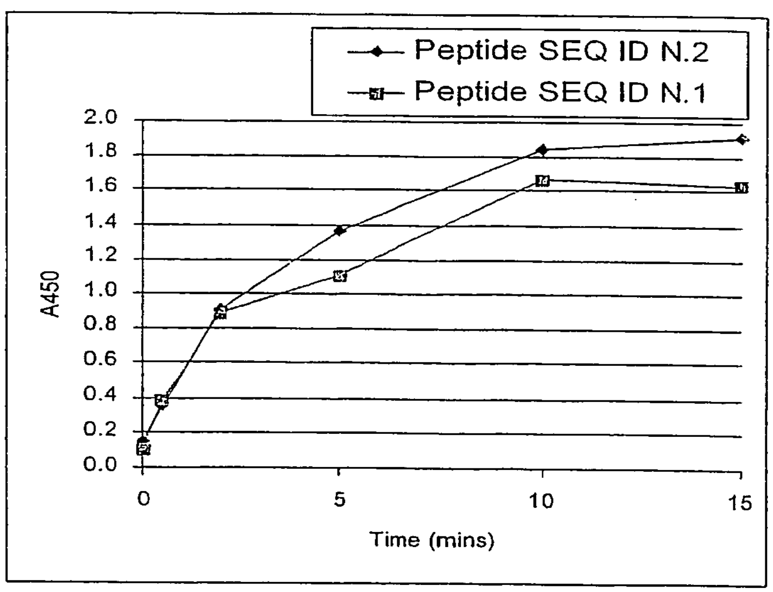
2.5
2
1.5
1
0.5
0
A450
No ALK
Plus ALK

B.


Peptide SEQ ID N.2
Peptide SEQ ID N.1
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1.8
1.6
1.4
1.2
1.0
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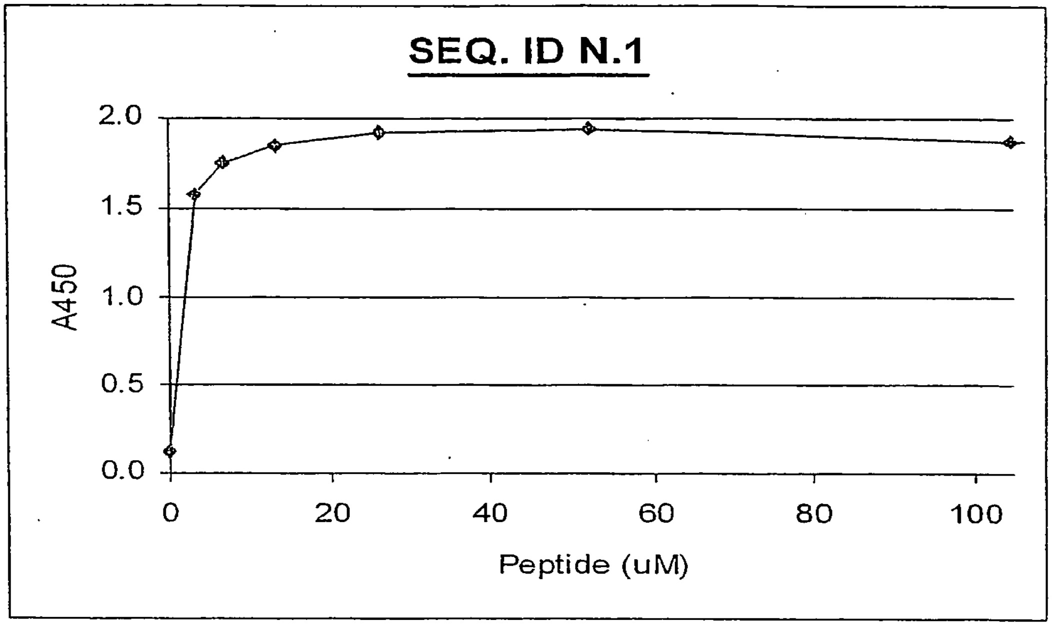
0.2
0.0
A450
0
5
10
15
Time (mins)

10/547842

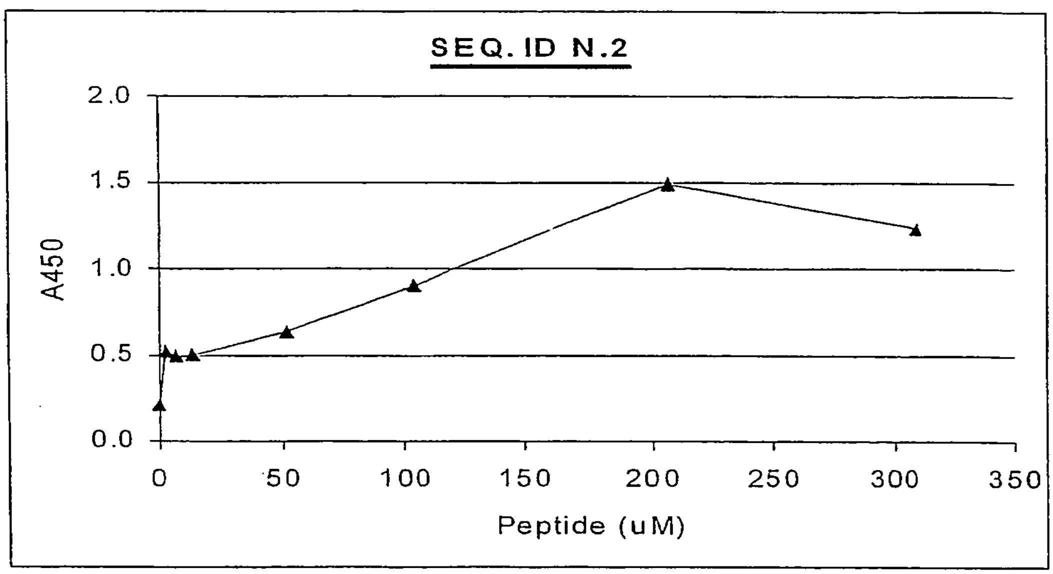


Fig 4

A.




B.



10/547842



Fig 5

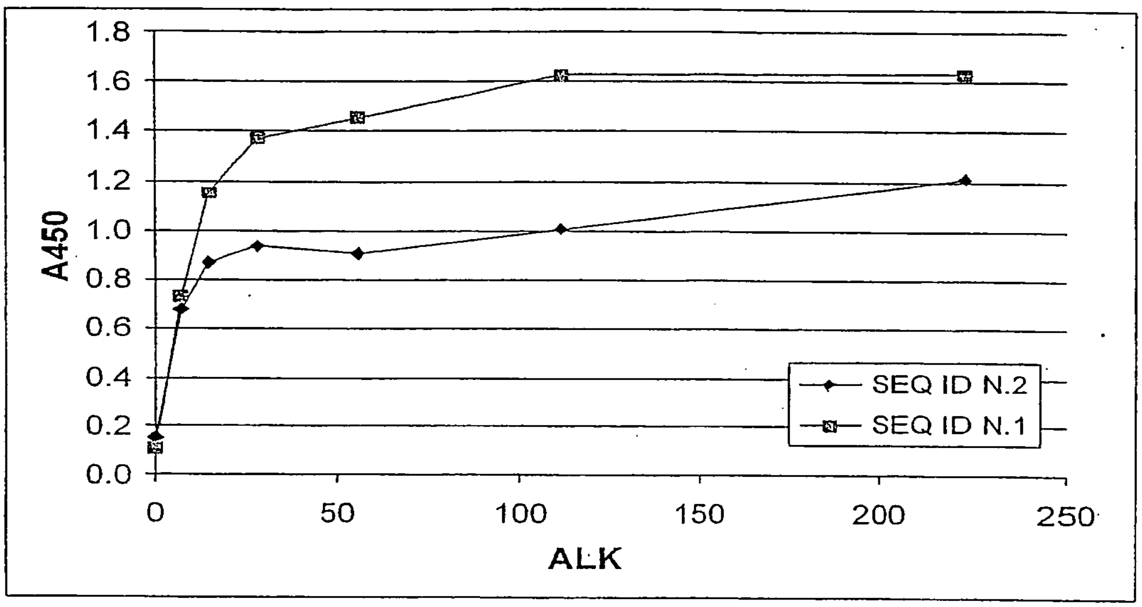
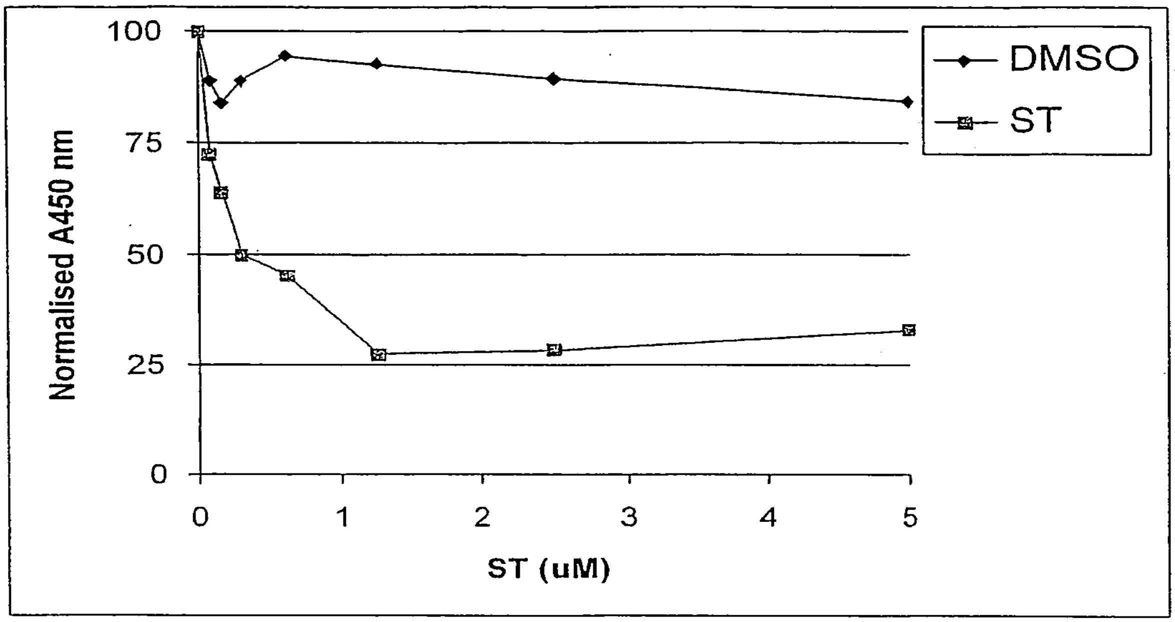


Fig 6



JC05 Rec'd PCT/PTO 02 SEP 2005

SEQUENCE LISTING

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<170> PatentIn version 3.1

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<212> PRT
<213> Unknown

<220>
<223> synthetic peptide

<400> 2

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1               5                   10                  15

Met Leu Pro Val Lys
            20
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# INTERNATIONAL SEARCH REPORT

Intern l Application No
PCT/EP2004/002185

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 C12Q1/48 C07K14/72 G01N33/74 A61K31/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE, Sequence Search

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | DATABASE WPI<br>Section Ch, Week 199325<br>Derwent Publications Ltd., London, GB;<br>Class A96, AN 1993-201126<br>XP002249142<br>& JP 05 126833 A (TOSOH CORP)<br>21 May 1993 (1993-05-21)<br>abstract<br>----- | 1-17,19,<br>20 |
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 July 2004 | 28 SEP 2004 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Gunster, M |

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# INTERNATIONAL SEARCH REPORT

## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-17, 19, 20

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-17,19,20

   ALK assay using a peptide of SEQ ID N. 1 or SEQ ID N. 2 and said peptides and related kits.

   ---

2. claim: 18

   Use of compounds of formula (I) for the preparation of a medicament for the treatment of ALK-related tumors.

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Intern ıl Application No
PCT/EP2004/002185

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Intern    l Application No
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Form PCT/ISA/210 (patent family annex) (January 2004)